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Case report

Illicit drugs in alternative biological specimens: A case report

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ABSTRACT

Postmortem tissues (e.g. liver, kidney) have been long used in forensic applications especially in those cases where blood is unavailable. The aim of this paper is to demonstrate the importance of the information provided to the forensic toxicologist at the time of carrying out the toxicological analysis, especially in cases where the samples commonly used in forensic toxicology are unavailable. This work describes the toxicological findings in a violent death resulting from a man who was hit by a train. Vitreous humor, liver and kidney were sent for toxicological analysis, once it was not possible to obtain blood and urine.

The validated procedures used in the routine casework of Forensic Toxicology Laboratory of the Centre Branch of the National Institute of Legal Medicine, were applied in the analysis of liver, kidney and vitreous humor, using gas chromatography–mass spectrometry after solid-phase extraction and gas chromatography-flame ionization detector for the analysis of drugs of abuse and ethanol, respectively. Morphine, codeine, cocaine, benzoylecgonine and ecgonine methyl ester were found in the liver and in the kidney and no ethanol was found in the vitreous humor.

The method validation included the study of specificity, selectivity, limits of detection, recovery and carryover. Although blood and urine are the most common and preferred matrices used for toxicological studies involving drugs of abuse, sometimes the choice of specimen is determined by the case under investigation. The forensic pathologist must be aware that relevant information must be provided so that the toxicological analysis can be conducted in accordance with case history, particularly when the only samples available for analysis are these "unconventional" specimens, since the interpretation of the obtained results is more difficult.

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1. Introduction

Forensic toxicology implies an understanding of drug use in the immediate antemortem setting, analytical methodologies and interpretation of results. However, in the field of postmortem forensic toxicology, the presence of interfering compounds in complex matrices is a challenge to accomplish reliable results, mainly when no directly comparable matrices are available. In these cases, the pretreatment of samples to separate drugs and metabolites from the matrices is crucial for the quality of the analysis.[1–4]

Over the last twenty years there has been a growing interest in the development of methodologies for qualitative and quantitative analysis of several drugs in postmortem matrices, despite the analysis of these specimens is limited by many factors of sample handling and processing including: putrefaction, autolysis, bacterial invasion, sample homogenization and complexity, difficulty in obtaining a representative sample, time-consuming techniques and analytical and chromatographic problems.[5–7] Thus, the analytical methods must be able to eliminate the presence of potentially interfering substances such as lipids, proteins and many other biomolecules usually present at high concentrations.[7,8]

Tissues like liver and kidney are often used in postmortem toxicology analysis, especially in those cases where blood is unavailable. These samples are suitable to prepare homogenates but, as already mentioned, they also contain high concentrations of lipids, which may interfere in the analytical procedures performance.

As a specimen, liver has the advantage of being relatively unaffected by postmortem redistribution compared to blood, although drug concentrations in the lobe proximal to the stomach may be affected by postmortem diffusion in cases of oral overdose.[7]

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Since most drugs are metabolized in this organ, both the parent substance and its metabolites may be present in this tissue in high concentrations. One of the major impediments to the use of liver in routine analysis is the lack of database information of liver concentrations, which complicates the interpretation of the results obtained. The kidney also assumes an important role in toxicological analysis once drugs and metabolites must pass through it to be excreted in the urine. As with other drugs and specimens, the process of interpretation should include consideration of all aspects involved in the cause of death investigation, including the analysis of multiple specimens.

This paper presents a particular case where the liver and kidney were the only available matrices to perform the toxicological analyses of drugs of abuse and also describes the analytical methods used to determine the substances found (morphine, codeine, cocaine, benzoylecgonine and ecgonine methyl ester).

2. Case report

A 36-year-old Portuguese male hit by a train was found dead on the railway line, with his limbs partially separated and several parts of his body exhibiting severe injuries.

The precise circumstances of his death were unknown and besides the clothes no other identification was found. At the scene of the accident there was a car with the key in the ignition and without any identification inside. The local authorities managed to identify its owner. The inquiry revealed that the car belonged to the victim. His family made a statement saying that he had not been seen for over three days and one of the parents mentioned the man's drug addiction.

Like in many other similar cases, blood and urine were unavailable. During the autopsy the pathologist collected vitreous humor, liver and kidney for toxicological analysis of alcohol and drugs of abuse.

3. Materials and methods

3.1. Reagents and standards

All drugs standards (morphine, codeine, 6-acetylmorphine, cocaine, benzoylecgonine, ecgonine methyl ester) as well as the trideuterated analogues of morphine (morphine-d_3) and benzoylecgonine (benzoylecgonine-d_3) were purchased from Cerilliant (Round Rock TX, USA) in solution at a concentration of 1 mg/mL.

Methanol (LiChrosolv®), 2-propanol, dichloromethane, n-hexane, ammonia solution 25%, sodium hydrogen phosphate and hydrogen carbonate, potassium dihydrogen phosphate, sodium and potassium chloride, sodium carbonate, phosphoric acid 10% and hydrochloric acid 32%, all of analytical grade, were purchased from Merck (Darmstadt, Germany).

N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and trimethylchlorosilane (TMCS) were purchased from Sigma–Aldrich (St. Louis, MO) and Macherey–Nagel (Düren, Germany), respectively.

Working solutions, of all drug standards, were prepared by dilution of the stock solutions with methanol. All solutions were protected from light and stored at a temperature between 2 and 8 °C.

3.2. Biological samples

Validation experiments were performed in blank liver and kidney samples obtained at the Forensic Pathology Service of the National Institute of Legal Medicine – Centre Branch, Coimbra, Portugal. These samples were free of drugs of abuse, as they were screened before being used for both calibrators and control samples.

The toxicological analysis of drugs of abuse was performed in the liver and kidney belonging to the deceased who was autopsied at the Forensic Pathology Service of the National Institute of Legal Medicine – Centre Branch, Coimbra, Portugal.

3.3. Chromatographic conditions

A HP 6890 gas chromatograph (Hewlett–Packard, Waldbronn, Germany) equipped with a 5973 mass-selective detector (Hewlett–Packard) and a capillary column (30 m × 0.32 mm i.d., 0.25 mm film thickness) with 5% phenylmethylsiloxane (HP-5 MS) from J&W Scientific (Folsom, CA, USA) was used. The chromatographic conditions were as follows: initial oven temperature was 90 °C for 2 min, which was increased by 20 °C/min to 300 °C and held for 3 min. The split injection mode was used at a ratio of 6:1, and helium was used as the carrier gas with a constant flow rate of 1.2 mL/min. The mass spectrometer was operated with a filament current of 300 μA at electron energy of 70 eV in the electron ionization (EI) mode. The temperatures of the injector and detector were set at 250 and 280 °C respectively. Confirmation was done in the selected ion monitoring (SIM) mode, and the ions were monitored at *m/z* 236, 429 and 414 for morphine; at *m/z* 371, 178 and 196 for codeine; at *m/z* 399, 340 and 287 for 6-acetylmorphine; at *m/z* 82, 182 and 303 for cocaine; at *m/z* 82, 240 and 361 for benzoylecgonine and at *m/z* 82, 96 and 271 for ecgonine methyl ester. For the internal standards, only one ion was monitored for each compound, at *m/z* 432 for morphine-d_3 and at *m/z* 243 for benzoilecgonine-$d_{3.}$

3.4. Samples preparation

Portions of liver and kidney (approximately 2 g) were placed into separate disposable plastic tubes and then ultrasonicated until homogeneity. After this, the samples were centrifuged at 3000 rpm for 5 min and the aqueous phase was collected to clean plastic tubes for the additional extraction steps. Drugs of abuse were extracted and derivatized as described below:

3.4.1. Opiates extraction

To the pretreated extracts was added 8 mL of phosphate buffered saline, after addition of 25 μL of morphine- d_3 (5 μg/mL) and subsequently the samples were homogenized for 10 min by rotation/inversion movements. The extraction of the opiates were performed with a Vac-Elut system assembled with columns Oasis® MCX (3 mL, 60 mg) purchased from Waters (Milford, MA, USA). The columns were conditioned with 2 mL of methanol and 2 mL of deionized water before applying the samples. After this, the columns were washed sequentially with 2 mL of carbonate buffer 0.15 M, 2 mL of deionized water, and 3 mL of *n*-hexane. Then, after drying under full vacuum for 15 min, the opiates were eluted with 2 mL of a mixture of dichloromethane: isopropanol: ammonia (78:20:2, *v*/*v*/*v*). The obtained extracts were evaporated to dryness at 40 °C under a gentle nitrogen stream.

3.4.2. Cocaine and metabolites extraction

To the pretreated extracts was added 8 mL of 0.1 M KH_2PO_4, after addition of 25 μL of benzoylecgonine –d3 (5 μg/mL) and subsequently the samples were homogenized for 10 min by rotation/inversion movements. The columns were conditioned with 2 mL of methanol and 2 mL of deionized water before applying the homogenates to the previously mentioned system. Then, the columns were washed sequentially with 2 mL of deionized water, 2 mL of hydrochloric acid 0.1 M, 2 mL of dichloromethane:

methanol (70:30, *v/v*), and 3 mL of *n*-hexane. After drying the columns under full vacuum for 15 min, the analytes were eluted with 2 mL of a mixture of dichloromethane: isopropanol: ammonia (78:20:2, *v/v/v*). The obtained extracts were evaporated to dryness at 40 °C under a gentle nitrogen stream.

3.4.3. Derivatization procedure

Dried elutes (for opiates and cocaine and metabolites) were dissolved in 60 μL of the derivatization reagent MSTFA with 5% of TMCS, and after vortex mixed derivatization occured at 80 °C for 30 min. Then, the derivatized elutes were transferred to the autosampler vials, and a 2 μL aliquot was injected into the chromatographic system.

3.5. Method validation

The described procedures are being used in routine analysis at the Forensic Toxicology Laboratory of the Centre Branch of the National Institute of Legal Medicine, for confirmation of opiates and cocaine and its metabolites in alternative samples. These procedures of confirmation were validated in terms of selectivity/specificity, limits of detection, repeatability and recovery in accordance with the internal validation guidelines of our laboratory. The methods were found to be selective, as no matrix interferences were observed at the retention times and at *m/z* values of the monitored ions, by analyzing blank samples from different origins. The limits of detection, defined as the lowest tested concentrations yielding a signal-to-noise ratio of at least 3, were 4, 5 and 4 ng/mL for morphine, codeine and 6-acetylmorphine respectively, and 3 ng/mL for cocaine, and 4 ng/mL for both benzoylecgonine and ecgonine methyl ester. The repeatability was evaluated at two concentrations levels for all compounds (100 and 1000 ng/mL) using eight spiked samples (for each concentration). The obtained CVs were lower than 5% for all compounds at both concentrations. The extraction efficiency was upper than 70% for cocaine and metabolites and upper than 91% for opiates at 100 ng/mL ($n = 4$) and 1000 ng/mL ($n = 4$). No carryover was observed for concentrations as high as 1000 ng/mL.

3.6. Acceptance criteria for compounds identification

The criteria for identification of compounds was established according to the recommendations of the World Anti-Doping Agency[9] as follows. For chromatography, the relative retention time of the substance must fall within a 1% window, or 0.2 min in absolute terms, from that of the same substance in a quality control sample prepared and analyzed contemporaneously. Mass-spectrometric identification must include at least three diagnostic ions, and their relative intensities should not differ by more than a tolerated amount from those generated by the same substance in a quality control sample prepared and analyzed contemporaneously (if the relative intensity of the ion is within a 25–50% interval of the base peak in the control sample, a maximum relative tolerance of ±20% will be allowed for the same ion in the sample; if this intensity is less than 25% or higher than 50% in the control sample, then absolute tolerances of ±5 and ±10%, respectively, will be allowed for the ion in the sample). It is also required, a signal-to-noise ratio greater than 3:1 for the least intense diagnostic ion.

3.7. Ethanol analysis

Aliquots of 0,1 mL of vitreous humor were diluted with 1 mL of n-propanol (internal standard solution at 0.1 g/L) in a 10 mL headspace vial and directly analyzed by headspace gas chromatography-flame ionization detection (GC-FID-HS). The oven was operated at a constant temperature of 40 °C and the injector and detector temperatures were set at 150 °C and 250 °C, respectively.

The calculated limits of detection and quantitation were 0,02 and 0.07 g/L, respectively.

4. Results and discussion

Ethanol was not detected in the vitreous humor. Cannabinoids, amphetamines and related compounds were not detected both in the liver and in the kidney.

Morphine, codeine, cocaine and its metabolites benzoylecgonine and ecgonine methyl ester were detected and confirmed in the liver and kidney. It was not possible to determine 6-acetylmorphine in the liver and kidney, probably due to its instability and transformation to morphine in the liver. It is known that within minutes of use, heroin is spontaneously and enzymatically deacetylated to 6-acetylmorphine which is then further hydrolyzed to morphine. Once in the liver, morphine is metabolized into other compounds. Since both heroin and codeine are metabolized to morphine, the detection of free or conjugated morphine in biological samples can result from the administration of morphine, codeine or heroin. Thus, several studies proposed the presence of 6-acetylmorphine in biological samples as a specific marker of heroin abuse.[10–15]

The concentration of the confirmed compounds were estimated on the basis of the peak area ratio between the quantifier ion of the substance (morphine *m/z* 429, codeine *m/z* 371, cocaine *m/z* 182, benzoylecgonine *m/z* 240 and ecgonine methyl ester *m/z* 82) and the internal standard (*m/z* 432 for the opiates and *m/z* 243 for the cocaine and its metabolites), and this was directly compared with the peak area ratios obtained from two control samples at 200 and 1000 ng/mL (Table 1).

The results obtained in this study revealed evidence of cocaine intake[12,16–18] and also a high probability of heroin intake. Both the illicit drug heroin and the prescription drug codeine are metabolized to morphine, however 6-acetylmorphine could not be found, which complicates the interpretation of heroin intake. Nevertheless, the high ratio of morphine to codeine concentrations, found in the liver and in the kidney, is an indicator of heroin use, not a prescribed medication with codeine.[14]

The lack of information provided both from the place where the victim was found and the circumstances of his death indicated the need for a toxicological analysis despite blood and urine unavailability. The use of alternative samples like liver and kidney is a great challenge to the forensic toxicologist, mainly due to the absence of directly comparable reference matrices. These biological specimens should not be seen as substitutes for traditional ones (blood and urine) but as complementary samples that can provide important information about the intake of drugs of abuse, or in those specific circumstances where the choice of sample is limited by the case under investigation.

This work demonstrates that under certain circumstances the forensic pathologist should collect a more comprehensive and meaningful alternative sample, as a substitute option for blood and urine, and provide all existing circumstantial information that can be useful in conducting the toxicological analysis.

Table 1
Concentrations of drugs determined in liver and kidney (ng/mL).

	Morphine	Codeine	Cocaine	Benzoylecgonine	Ecgonine methyl ester
Liver	1098	79	80	1674	1015
Kidney	1162	127	316	2477	2308

Conflict of interest

There is no conflict of interest in this paper.

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Ethical approval

This study has been carried out in accordance with ethical rules and it is has not been submitted to Ethical Approval because it is a case report of a dead person in which no identification of the individual was given.

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